Table III—Steady-State Flux of Hydrocortisone from Solutions and Gels Containing 0.2% Hydrocortisone and 40% 2-Propanol

Polysorbate 80, % (w/v)	Formulation	Mean Flux ± SD, μg/hr/cm²
0	Solution	0.214 ± 0.037
0	Gel	0.239 ± 0.038
0.51	Solution	0.377 ± 0.008
0.51	Gel	0.367 ± 0.024

presented in this paper. From the intercept of the linear portion of the penetration plots with the abscissa, the hydrocortisone diffusion coefficient in the membrane was calculated (10) to be about 10^{-12} cm²/sec. The hydrocortisone diffusion coefficient in water is about 4.6×10^{-6} cm²/sec (19). Although the diffusion coefficient in the donor solutions was probably somewhat larger or smaller than this figure, it is clear that the hydrocortisone diffusion rate across the skin was much slower than that of the drug in the unstirred donor phase.

In some experiments, gels of practically the same composition were used in place of the hydrocortisone solutions. The gels differed from the solutions only in that they contained 1% hydroxyethylcellulose as a gelling agent. There was no significant difference in the penetration rate between the gels and the solutions (Table III). This result was true whether polysorbate 80 was included or not. The increased viscosity of the solution on the addition of the gelling agent did not influence penetration significantly. This finding might be anticipated since changes in macroscopic viscosity in a gel system usually do not lead to marked changes in the diffusion coefficient of a drug dissolved in the gel (20). Therefore, with the gels as with the solutions, hydrocortisone transport through the skin was rate limiting and the relatively minor changes in the hydrocortisone diffusion coefficient in the donor did not affect the flux.

An interesting application of this phenomenon is that preliminary formulation work involving percutaneous absorption can be conducted using simple solutions of the drug in a device similar to the cell used in this study. If the finished dosage form is to be a gel, the preliminary results should be useful indicators of how the gel will perform.

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Rapid Blue Tetrazolium Procedure for Analysis of Corticosteroids in Pharmaceutical Preparations

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Received August 22, 1977, from the **Dallas District, Food and Drug Administration, Dallas, TX 75204,* and the ‡*Department of Chemistry, Southern Methodist University, Dallas, TX 75275.* Accepted for publication September 23, 1977.

Abstract □ A rapid quantitative analysis of nine selected corticosteroids and corticosteroid esters at room temperature is described. The procedure is similar to the official blue tetrazolium reaction for corticosteroids, except that methylene chloride instead of alcohol USP is used as a solvent and the reagents are dissolved in or diluted with nonaqueous solvents. These two modifications reduce the medium polarity, which increases the reaction rate. The reactions are complete in 7–18 min, and the formazans are stable for at least 90 min. The results from 15 different pharmaceutical formulations, 12 containing hydrocortisone and three containing prednisolone acetate, show that the proposed method gives results that compare favorably with those obtained by the official blue tetrazolium, isoniazid, and phenylhydrazine procedures.

Keyphrases □ Corticosteroids, various—blue tetrazolium spectrophotometric analyses in pharmaceutical preparations □ Blue tetrazolium—reaction with various corticosteroids, spectrophotometric analyses in pharmaceutical preparations □ Spectrophotometry—analyses using blue tetrazolium reaction, various corticosteroids in pharmaceutical preparations

The blue tetrazolium reaction is widely used for the analysis of corticosteroids. USP XIX (1) and NF XIV (2) use a slightly modified procedure of Mader and Buck (3) for corticosteroid analysis. Blue tetrazolium (I), 3,3′-(3,3′-dimethoxy-4,4′-biphenylylene)bis(2,5-diphenyl-2*H*-tetrazolium chloride), oxidizes the α-keto moiety of the C_{17} side chain of corticosteroids in strongly alkaline solution (4) and is reduced quantitatively to a highly colored formazan whose concentration is measured spectrophotometrically.

A recent paper (5) showed that the reaction rate of I with corticosteroids was inversely proportional to the dielectric constant and directly proportional to the hydrogen bonding ability of the solvent. Furthermore, the substitution of 20.0 ml of methylene chloride for alcohol USP in the I assay for steroids greatly speeds the reaction while the quantitative nature and specificity of the compendial procedure (1, 2) are retained. The monographs for various corticosteroids utilizing I as the official procedure require a 45–90-min color development time along with various special techniques such as that for flurandrenolide (6) which requires a water bath maintained at 40 ± 1°, accurately timed intervals, and subsequent quenching of the reaction with glacial acetic acid.

The data presented in this paper show that the reaction of I with nine selected steroids in methylene chloride is quantitative, up to six times as rapid as in alcohol USP, essentially complete in 15 min, and requires no special handling. Analysis of 15 different commercial formulations shows that the results obtained with the proposed procedure compare favorably with the results obtained by the official I procedure (1) as well as the Umberger isoniazid (7) and the Silber–Porter (8) reactions.

EXPERIMENTAL

Apparatus—The following were used: UV–visible recording spectrophotometers[1] with 1-cm stoppered quartz cells, glass chromatographic columns for partition chromatography (2.2 × 25 cm constricted at one end to 0.4 × 5 cm), an aluminum tamping rod, an electrobalance[2], and TLC equipment[3].

Materials—Alcohol USP, analytical reagent grade absolute ethanol, and distilled-in-glass grade acetonitrile, chloroform, *n*-heptane, absolute methanol, and methylene chloride were used along with blue tetrazolium[4], 10% aqueous tetramethylammonium hydroxide[5], and acid-washed diatomaceous earth[6]. Also used were USP reference standard cortisone acetate, hydrocortisone, hydrocortisone acetate, prednisolone acetate, and prednisone; NF reference standard dexamethasone, fluprednisolone, and flurandrenolide; cortisone[7]; and dihydrocortisone[8].

Reagents—A 1% solution of tetramethylammonium hydroxide was prepared by diluting 5.00 ml of the 10% aqueous solution to 50.0 ml with absolute ethanol. Blue tetrazolium, 5 mg/ml, was prepared by dissolving 50.0 mg in 10.0 ml of absolute methanol. Standard corticosteroid solutions were prepared to contain 0.010 mg of steroid/ml in methylene chloride, unless otherwise indicated.

Mutually saturated acetonitrile–*n*-heptane was prepared as follows. Acetonitrile, 25 ml, was mixed with 300 ml of *n*-heptane (sufficient for two determinations) in a separator, agitated vigorously for 2 min, and allowed to stand until both layers were clear. These mutually saturated solutions were used whenever acetonitrile or *n*-heptane is indicated in these directions.

Sample Preparation—*Suspensions*—After the suspension was mixed well, duplicate samples were taken immediately with 5-ml pipets (T.C.) and drained into separate 100-ml beakers. The pipets were washed twice with 0.5-ml portions each of acetonitrile, methanol, and *n*-heptane into the same beakers, and the procedure was continued as directed under *Column Preparation.*

Creams and Ointments—A composite of several containers was prepared, and a sample of about 5 g was weighed accurately into a beaker. Methanol, 30 ml, was added, and the mixture was heated on the steam bath with periodic agitation to incipient boiling to dissolve the sample. It was then cooled in an ice bath until the residue solidified, and the liquid was decanted into a 100-ml volumetric flask. The extraction was repeated three times with 20-ml portions of methanol.

The combined sample extracts were adjusted to room temperature and diluted to volume with methanol. An accurately measured aliquot containing about 1 mg of corticosteroid was evaporated to dryness carefully. The sample residue was dissolved in 1.5 ml each of acetonitrile and *n*-heptane for cleanup as directed under *Column Preparation.*

Lotions—The contents of several containers were mixed well and centrifuged at 400 rpm for 20 min. From 1.0 to 5.0 ml of sample, depending on the concentration, was transferred to a 100-ml flask by pipet (T.C.). The pipet was washed with methanol, and the sample was diluted to volume with methanol. An accurately measured aliquot, equivalent to about 1 mg of corticosteroid, was evaporated carefully just to dryness on the steam bath. The sample residue was dissolved in 1.5 ml of acetonitrile and 1.5 ml of *n*-heptane for cleanup as directed under *Column Preparation.*

Gels—A composite of several containers was mixed well, and a sample of about 2.5 g, accurately weighed, was made up to 100.0 ml with alcohol USP. An accurately measured aliquot, equivalent to about 1 mg of corticosteroid, was evaporated carefully just to dryness on the steam bath. The sample residue was dissolved in 1.5 ml of acetonitrile and 1.5 ml of *n*-heptane for cleanup as directed under *Column Preparation.*

Column Preparation—*Acetonitrile Layer*—A glass wool plug was inserted in the bottom of a chromatographic column. Then a 4-g portion of diatomaceous earth was thoroughly mixed with 4.0 ml of acetonitrile, transferred to the column, and packed firmly with a tamping rod.

Sample Layer—The sample solution, prepared as directed under *Sample Preparation,* was mixed thoroughly with 3 g of diatomaceous earth to yield a light fluffy mixture. It was then transferred to the column above the acetonitrile layer and packed firmly. The sample beaker, tamping rod, spatula, and funnel were dry washed with about 1 g of diatomaceous earth, which was added to the column. The same equipment was dry washed with glass wool, which was placed on top of the sample layer plus washings and packed firmly. The beaker was retained and washed with the *n*-heptane and chloroform used during column elution.

The sample beaker was washed with 150 ml of *n*-heptane in small portions and transferred to the column to maintain a liquid head approximately 12 cm above the column bed. The last wash was allowed to drain completely from the column, the tip was rinsed with alcohol USP, and the entire effluent was discarded. A new beaker was placed under the column, and the sample beaker was washed with 125 ml of chloroform in small portions, which were added to the column to maintain the liquid level close to the top of the column.

The last portion was allowed to drain completely, and the tip was rinsed with alcohol USP. The effluent was evaporated carefully just to dryness on a steam bath under a hood to ensure complete removal of the acetonitrile. The residue was dissolved and diluted accurately to volume with methylene chloride, which contained approximately 0.010 mg of corticosteroid/ml.

Isoniazid Method—The procedure of Umberger (7) was used, except that the hydrochloric acid concentration was doubled to increase the sensitivity.

Phenylhydrazine Method—The procedure of Silber and Porter (8) was followed.

Blue Tetrazolium Method—The procedure given in USP XIX (1) was followed.

Proposed Method—Aliquots of 20 ml were run by the I procedure given in USP XIX (1), except that the tetramethylammonium hydroxide reagent was diluted with absolute ethanol instead of alcohol USP, the blue tetrazolium reagent was prepared with absolute methanol in place of alcohol USP, the spectrophotometric scans from 720 to 490 nm were made against methylene chloride as reference instead of the reagent blank, and the scans were started 15 min after tetramethylammonium hydroxide addition. The scans were always made in the order of reagent blank, standard, sample, and reagent blank, and the reagent blank was kept in the dark between the original and final scans. The absorbance was read from the absorbance maximum at about 525 nm.

Each measured absorbance value had to be corrected for the continuously increasing reagent blank absorbance. The correction for each reading was obtained by dividing the increase in absorbance of the reagent blank by the number of scans (omitting the original reagent blank scan) to obtain an average increase per scan. This average was then multiplied by the scan number and added to the original reagent blank absorbance. The net absorbance was then calculated by subtracting the corrected reagent blank absorbance from the measured absorbance for that scan.

Replication Studies of Absorbances Produced by Proposed Blue Tetrazolium Method—Accurately weighed portions of each of the 10

[1] Cary models 15 and 17.
[2] Cahn model G-2.
[3] Eastman 6060 silica gel.
[4] Dajac Laboratories.
[5] Eastman Organic Chemicals.
[6] Celite 545, Johns-Manville Product Corp.
[7] K and K Laboratories.
[8] Mann Research Laboratories.

Table I—Absorbance Replication of Proposed Procedure

Run	Cortisone	Cortisone Acetate	Dexamethasone	Dihydrocortisone Acetate	Fluprednisolone	Flurandrenolide	Hydrocortisone	Hydrocortisone Acetate	Prednisolone Acetate	Prednisone
1[a]	0.629	0.569	0.575	0.535	0.580	0.521	0.584	0.544	0.512	0.568
2	0.628	0.574	0.578	0.535	0.592	0.516	0.584	0.531	0.511	0.571
3	0.630	0.575	0.586	0.535	0.580	0.518	0.586	0.534	0.511	0.577
4	0.630	0.572	0.585	0.534	0.591	0.521	0.587	0.539	0.511	0.574
5	0.631	0.573	0.584	0.536	0.587	0.528	0.586	0.536	0.516	0.573
6	0.623	0.574	0.579	0.536	0.581	0.530	0.584	0.538	0.519	0.576
7	0.627	0.571	0.579	0.538	0.592	0.534	0.582	0.542	0.516	0.580
8	0.627	0.573	0.579	0.540	0.585	0.538	0.586	0.549	0.509	0.580
9	0.617	0.571	0.587	0.536	0.581	0.532	0.587	0.542	0.514	0.581
10	0.617	0.572	0.581	0.543	0.582	0.539	0.587	0.543	0.515	0.579
11	0.627	0.572	0.586	0.526	0.581	0.536	ND[b]	0.538	ND[b]	0.578
12	ND[b]	0.569	0.587	0.541	0.583	ND[b]	ND[b]	0.541	ND[b]	0.581
Average	0.626	0.572	0.582	0.536	0.585	0.528	0.585	0.540	0.513	0.576
SD[c]	0.0046	0.0020	0.0040	0.0056	0.0040	0.0076	0.0016	0.0059	0.0033	0.0043
RSD, %	0.73	0.35	0.69	1.0	0.68	1.4	0.27	1.1	0.64	0.75

[a] Standards were run consecutively in the order 1–12. [b] ND = not determined. [c] Calculated from the range by the method of Dean and Dixon (9).

corticosteroid standards were dissolved in and diluted to 250.0 ml with methylene chloride to yield solutions containing approximately 0.010 mg/ml. Ten to 12 20.0-ml aliquots of each were analyzed by the proposed method, and the absorbances were determined (Table I). All solutions were kept in the dark during color development. The relative standard deviation, calculated from the range (9), varied from 0.35 to 1.4% with an overall average of 0.76%.

Beer's Law Study—More concentrated solutions, up to 3.5 mg/100 ml, were prepared for each of the 10 standards in methylene chloride. Aliquots of 2.00 or 3.00, 5.00, 10.0, 15.0, and 20.0 ml of each solution were taken, diluted to 20.0 ml with methylene chloride, and put through the proposed method. Absorbances ranged from 0.090 to 1.867, and correlation coefficients of 0.9993 or better were obtained for each steroid using a least-squares fitting program.

Color Development Time and Formazan Stability—A 20.0-ml aliquot of each of the 10 standards was tested according to the proposed method, except that the solution was transferred to a cell immediately after tetramethylammonium hydroxide addition. Readings were taken every minute *versus* a reagent blank for 90 min, and the time necessary to reach maximum absorption was noted for each steroid. The results (Table II) show that completion of the reaction, except for flurandrenolide, requires 7–18 min and that the formazan is stable for at least 90 min after reagent addition.

RESULTS AND DISCUSSION

The results of the analyses of 15 different pharmaceutical preparations containing corticosteroid and corticosteroid esters by the proposed procedure are shown in Table III. Duplicate analyses were run for each sample by four different methods. The results obtained by the proposed method compare favorably with those achieved by the official I procedure (1). Results of analyses obtained by the isoniazid and phenylhydrazine methods also are in agreement with those of the two I procedures, with the exception of Sample 8. The higher values obtained by the isoniazid method, which measures the amount of conjugated ketone in ring A of corticosteroid molecules, indicates possible decomposition of the C_{17} side chain in this sample. Interference to the I reaction by both methods in Sample 5 was confirmed by the method of variation of absorbance with time previously reported (10). Averages for all determinations of all samples by the four methods (Table III) show excellent agreement.

Table II—Reaction Time and Stability of Formazan in Methylene Chloride

Steroid	Time to Maximum Absorbance, min	Absorbance			Absorbance per Micromole[b]
		Original Maximum[a]	60 min	90 min	
Cortisone	10	0.670	0.682	0.685	1.045
Cortisone acetate	11	0.549	0.549	0.543	1.000
Dexamethasone	11	0.530	0.548	0.548	1.028
Dihydrocortisone acetate	13	0.570	0.578	0.577	1.043
Flupredniso lone	12	0.610	0.622	0.622	1.031
Flurandrenolide[c]	—	—	0.548	0.570	—
Hydrocortisone	18	0.579	0.590	0.596	1.039
Hydrocortisone acetate	13	0.620	0.629	0.627	1.056
Prednisolone acetate	16	0.509	0.510	0.509	1.020
Prednisone	7	0.583	0.571	0.563	1.019

[a] The absorbance noted at the time specified in the preceding column. [b] See Ref. 11 for definition. The average was 1.031. [c] The absorption maximum was not reached in 90 min.

Because of the low solubility of water in methylene chloride, it is necessary to substitute water-free solvents for alcohol USP for the dilutions of tetramethylammonium hydroxide and for the preparation of the I reagent for use in the proposed procedure to prevent the formation of liquid emulsions. Furthermore, the presence of small amounts of dissolved water increases the polarity of the solvent medium and increases the time required for the reaction to reach completion. A recent paper (5) showed that an increase in water content from 0.9 to 1.2% increased the time required for the hydrocortisone reaction with I to reach maximum absorbance from 13 to 90 min.

Beer's law was obeyed between 0.5 and 0.50 mg/24 ml for all 10 corticosteroids.

Reaction of I with flurandrenolide for 15 min produced an absorbance of 0.486, which is equivalent to 1.034 absorbance units/μmole or one reduction unit (11). This result indicates that the oxidation of the α-keto group was complete in 15 min, even though the absorbance continued to rise for 90 min. The increase is similar to the reaction of triamcinolone (11) and indicates the release of a second reduction unit through the slow hydrolysis of the cyclic diacetal with the acetone group of flurandrenolide. Apparently, the hydrolysis rate of the cyclic diacetal is the rate-limiting step in this reaction, and the absorbance increase could be expected to continue until hydrolysis is complete. The proposed method can be used for flurandrenolide and similar molecules by quenching the reaction by the addition of acetic acid to the blank, standard, and sample in rapid succession 15 min after the addition of tetramethylammonium hydroxide, at which time oxidation of the $C_{20,21}$ α-keto group is complete.

The rate studies (Table II) show that the reaction of I with nine selected corticosteroids and corticosteroid esters in methylene chloride was complete at room temperature in relatively short periods—from 7 to 18 min. Once the reaction was complete, the formazan was stable for at least 90 min from the time of tetramethylammonium hydroxide addition. The average value calculated in absorbance units per micromole produced from the reaction of I with the nine corticosteroids (Table II) was 1.031 in methylene chloride, identical to the average value of 1.031 previously reported (11) for 21 corticosteroid reactions with I in alcohol USP. Agreement of these values indicates that the reaction of these corticosteroids with I in methylene chloride is quantitative and is as sensitive as the comparable reaction in alcohol USP.

While the I reaction with steroids is complete in the specified time and the formazan produced is stable, the absorption of the blank continues to increase with time. The rate of absorbance increase in the light-protected blank is slower than the rate of absorbance increase of the blank in the reference cell when a long series of scans is necessary. As a consequence, the procedure described in USP XIX (1), which requires that the samples be scanned *versus* a reagent blank, will cause an error of approximately 0.001 absorbance unit/min when a long series of scans is made. The correct net absorbance for the samples can be obtained only

Table III—Analysis of Typical Pharmaceutical Formulations

Sample	Type of Sample[a]	Amount Declared, %	Found, % of Declared: Blue Tetrazolium, Proposed Method	Blue Tetrazolium, USP XIX Method	Isoniazid	Phenylhydrazine
1	Cream	0.25	95.0, 97.8	91.2, 91.7	96.0, 95.6	91.5, 90.8
2	Cream	0.5	96.7, 101.4	96.6, 100.0	94.1, 97.7	93.8, 98.5
3	Cream	1	97.1, 97.6	99.2, 98.6	98.6, 99.6	96.1, 100.3
4	Gel	1	97.5, 99.9	97.5, 97.4	99.9, 97.9	99.5, 97.9
5	Lotion	0.25	97.1, 96.9	96.2, 97.2	92.8, 88.1	ND[b]
6	Lotion	0.5	100.0, 95.9	99.5, 96.3	ND[b]	101.2, 98.8
7	Lotion	0.5	99.8, 99.2	99.6, 99.2	100.9, 101.3	99.2, 100.4
8	Lotion	0.125	90.5, 89.0	92.4, 92.8	102.2, 99.3	97.9, 95.8
9	Lotion	0.25	95.4, 96.6	96.3, 98.0	98.5, 97.8	95.2, 96.9
10	Lotion	0.5	98.7, 99.0	102.4, 102.6	101.9, 101.2	102.9, 102.3
11	Lotion	1.0	103.2, 104.4	106.4, 106.6	101.1, 101.3	103.2, 103.2
12	Lotion	1.0	94.3, 92.6	94.4, 91.2	92.5, 91.0	93.5, 94.6
13	Ointment[c]	0.25	98.6, 97.3	94.9, 94.7	98.8, 98.0	97.5, 98.5
14	Suspension[c]	0.25	89.3, 91.2	87.9, 89.8	88.4, 87.8	86.9, 92.7
15	Suspension[c]	0.25	71.6, 73.6	73.3, 74.8	74.0, 72.3	72.3, 73.5
	Average		95.2	95.3	95.3	95.6

[a] The steroids were hydrocortisone in Samples 1–12 and prednisolone acetate in Samples 13–15. [b] ND = not determined. [c] Product also contained 10% sodium sulfacetamide.

by scanning against the solvent and calculating the corrected net absorbance as shown under *Experimental*.

Methylene chloride solutions undergo evaporative concentration from glass-stoppered volumetric flasks at a daily average rate of approximately 0.1%[9] (v/v). This result indicates that the same standard could be used for several days without significant change. In this study, however, the standards were used only on the day of preparation.

In some cases, the corticosteroid was so slowly soluble in methylene chloride that it was necessary to let it stand for 1 hr with periodic agitation or place it in an ultrasonic instrument for not more than 30 sec to ensure complete solution.

The proposed procedure for the analysis of corticosteroids with I is rapid and quantitative. In most cases, the reaction is complete within 15 min after addition of the tetramethylammonium hydroxide reagent and the formazans produced are stable for at least 90 min. Analysis of 15 different pharmaceutical products for corticosteroids by the proposed I procedure gave results that compared favorably with those obtained by three different analytical methods.

[9] Unpublished work.

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Preferential Localization of Radiolabeled Liposomes in Liver

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Abstract □ Liposome formulations were studied to achieve an efficient entrapment procedure for the production of liposomes of ^{99m}Tc-pentetic acid. The entrapment efficiency was studied by separation of the product using column chromatography. The particle-size range of the prepared liposomes was evaluated using electron microscopy. Entrapment techniques and separation procedures led to a liposome preparation with particles in the colloidal size range (0.001–0.5 μm). Dramatic differences in the organ distribution of the liposome preparation in mice were produced when different particle-size ranges were injected. Liposomes eluted in the first fraction after the void volume led to a maximum uptake by the liver and spleen 10 min after intravenous injection. Liposomes from pooled fractions provided less than half of the activity in the liver, as did the narrow size range liposome preparation.

Keyphrases □ Liposomes, radiolabeled—^{99m}Tc-pentetic acid, organ distribution in mice, effect of particle size □ Radiolabeled liposomes—^{99m}Tc-pentetic acid, organ distribution in mice, effect of particle size □ ^{99m}Tc-Pentetic acid liposomes—organ distribution in mice, effect of particle size □ Distribution, organ—^{99m}Tc-pentetic acid liposomes in mice, effect of particle size □ Particle size—^{99m}Tc-pentetic acid liposomes, effect on organ distribution in mice

Liposomes have been described as minute vesicles composed of lipid bilayers (1). Spontaneous formation of liposomes occurs when a combination of certain lipids is dispersed throughout an aqueous solution (2). Materials dissolved in the aqueous solution become trapped in the enclosed aqueous compartments, which form in an alter-